Spatial BioSystems

Multiplexer V1

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Instruction Manual

Content

Note: This manual is for prototype 8 and might not be applicable to other designed protoypes.

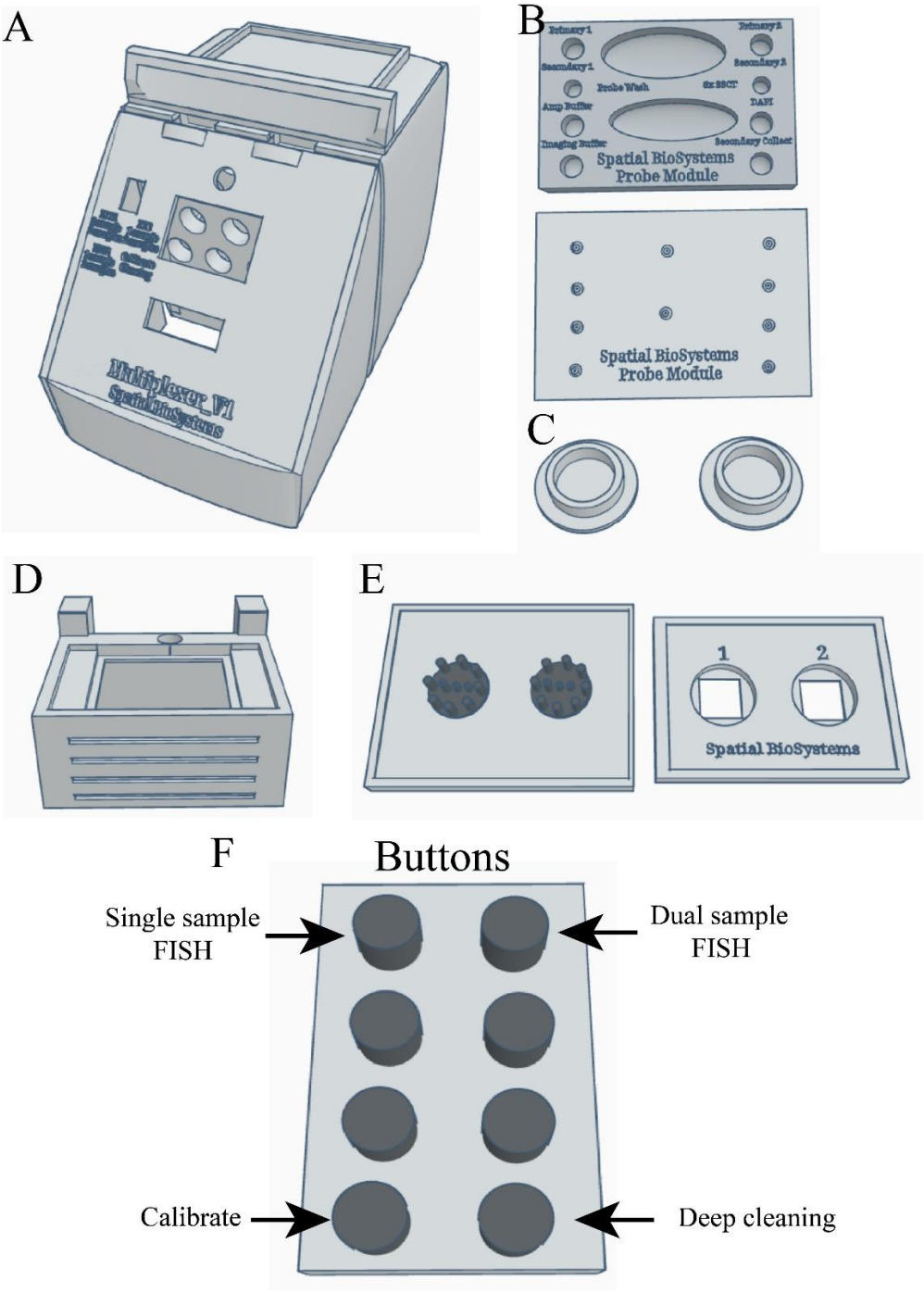

Figure 1: Main parts of the system. (**A**) Main body, (**B**) probe module, (**C**) sample plates, (**D**) reactor base, (**E**) sample holders, and (**F**) buttons.

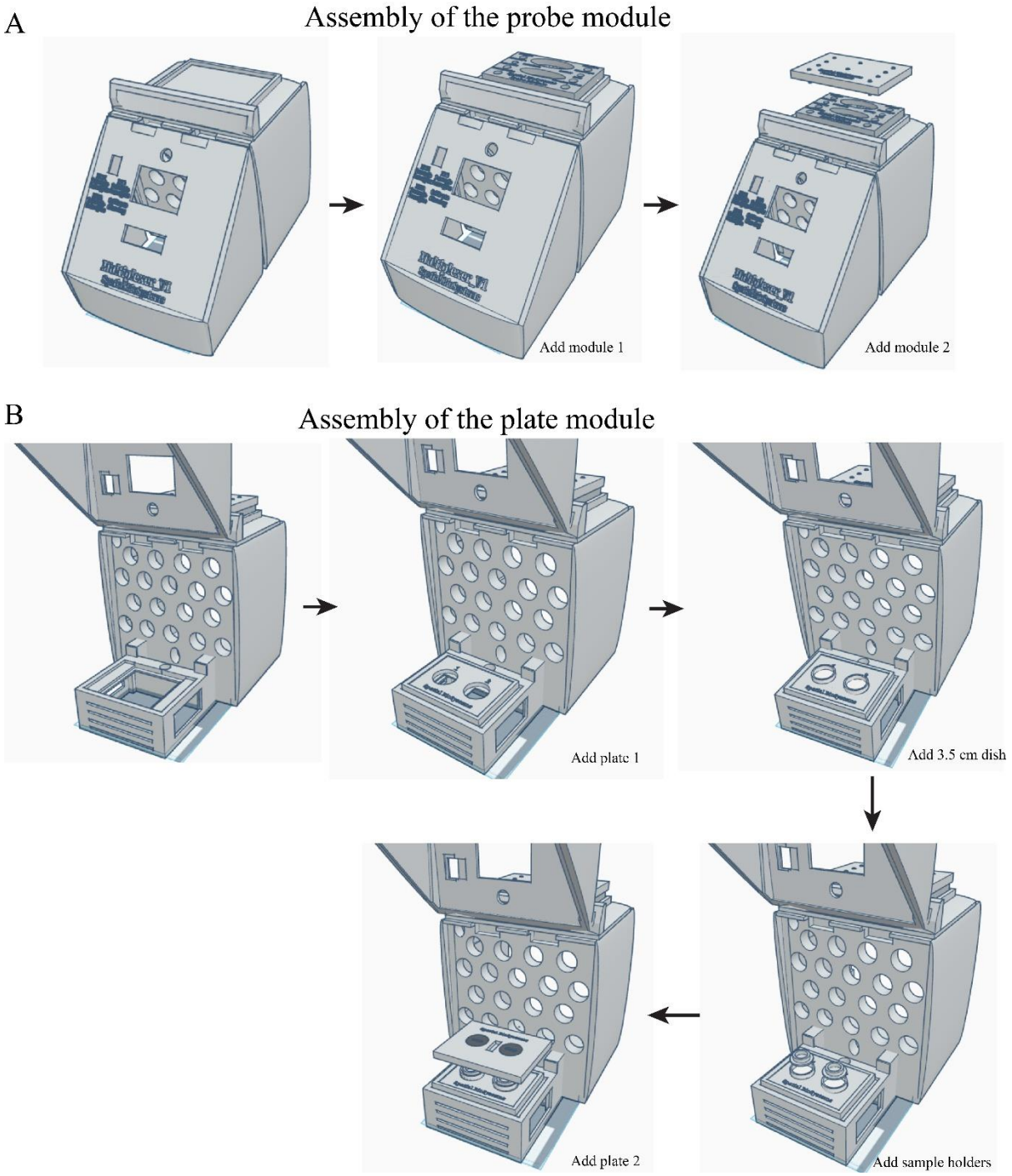

Figure 2: Assembly instruction. (**A**) Assembly of the probe modules. (**B**) Assembly of the plate module.

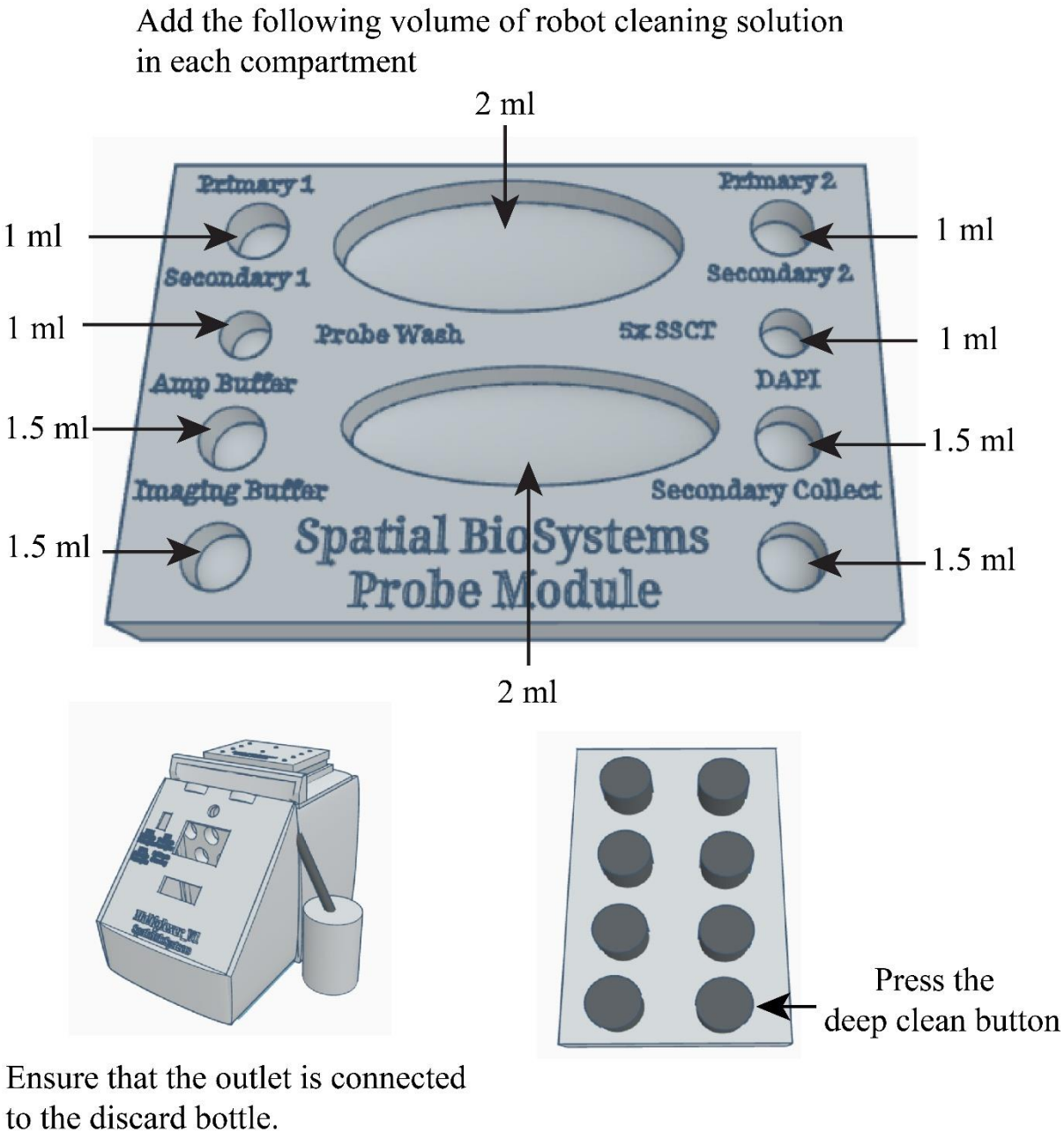

Figure 3: Deep cleaning protocol. Add the displayed volume of the robot cleaning solution, ensure that the outlet is connected to the discard bottle, and press the deep clean button (button 8). Repeat the step using molecular grade/DEPC water.

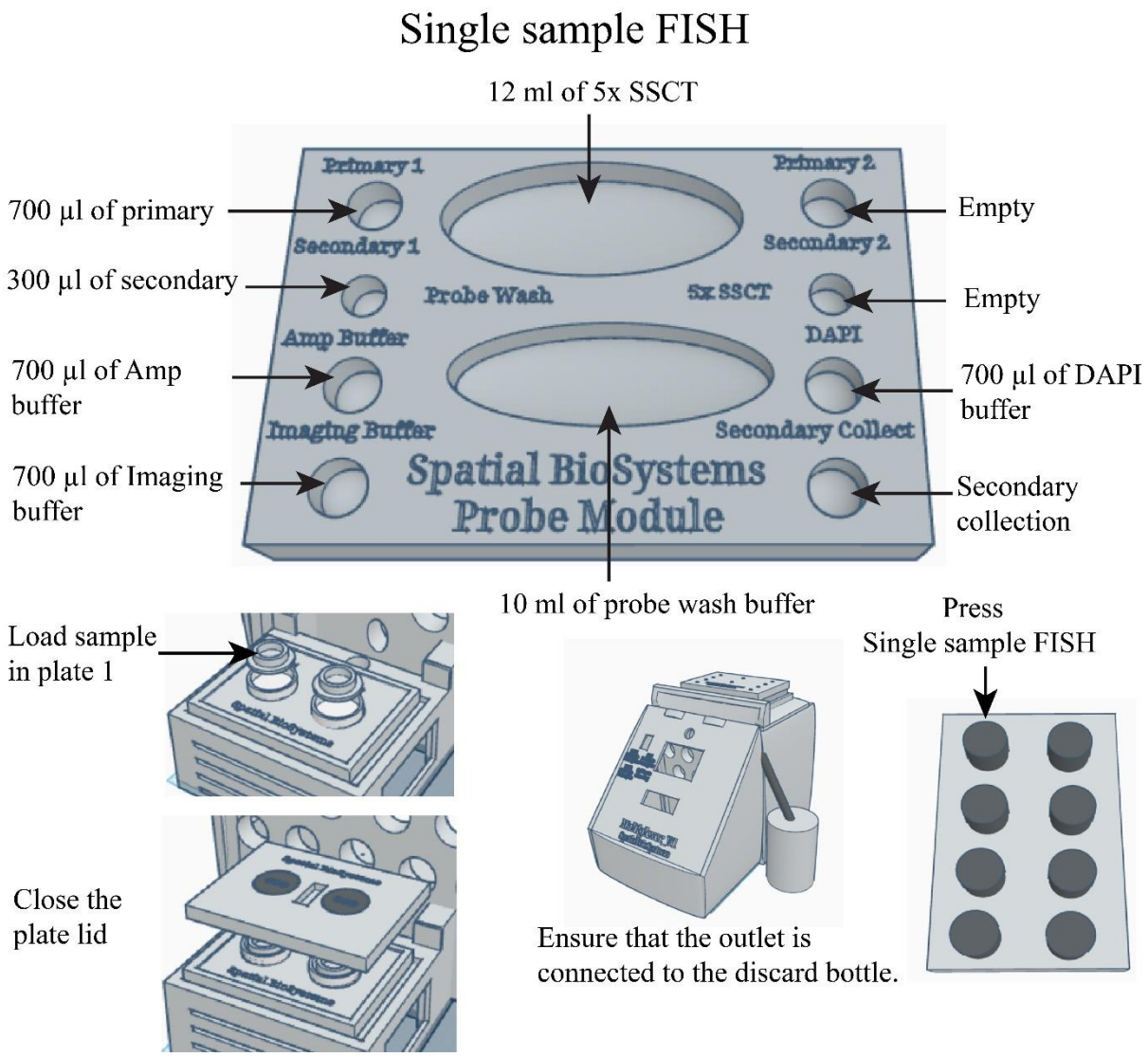

Figure 4. Single sample FISH instruction. Wash the probe module plate using DEPC water and load the buffers, sample, and press button 1 (single sample FISH)

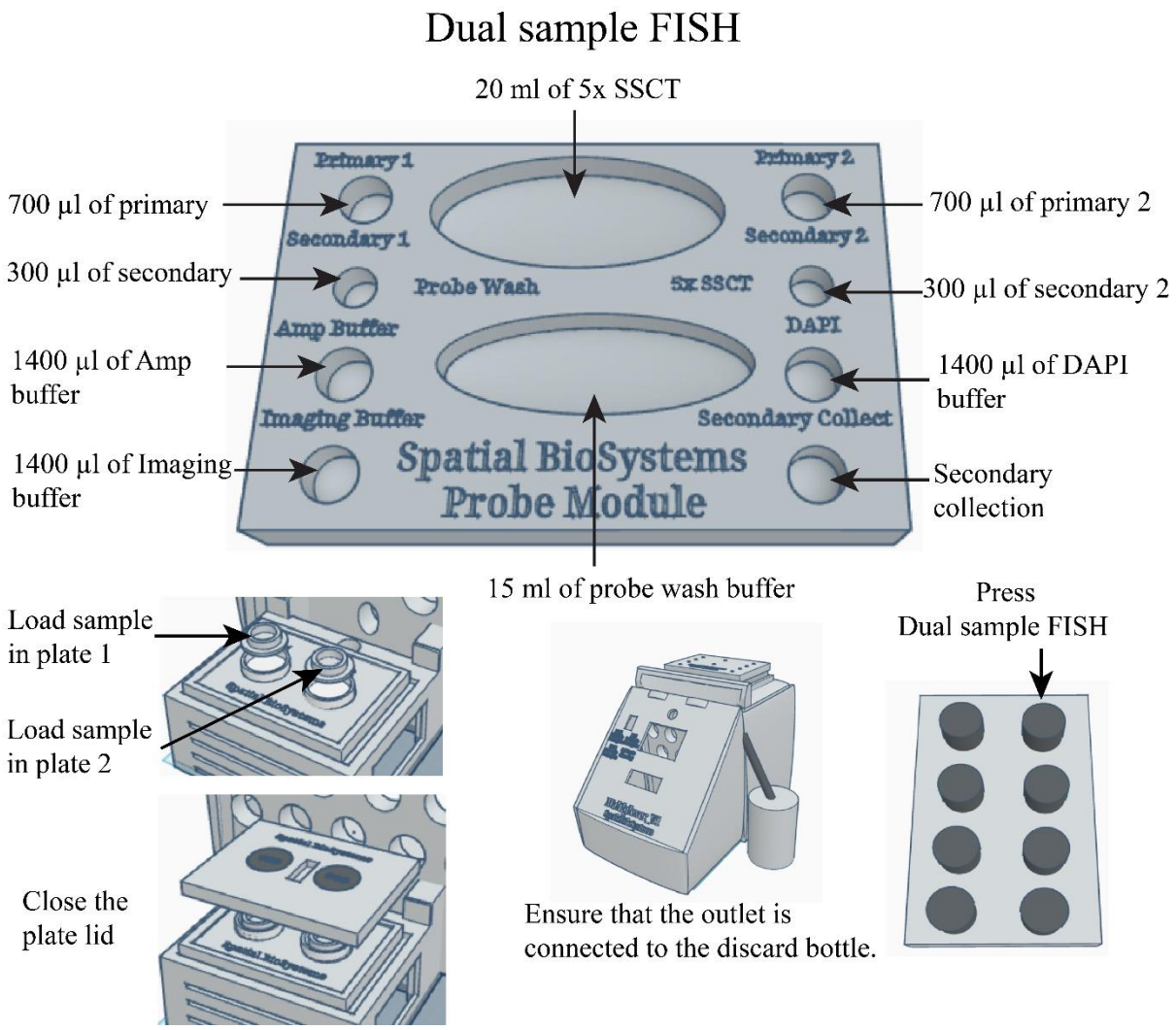

Figure 5: Dual sample FISH instruction. Wash the probe module plate using DEPC water and load the buffers, samples, and press button 2 (dual sample FISH)

Basic troubleshooting

1) Clogged outlet: In rare cases the outlet tube can get clogged with salts and samples. Perform a simple wash using a pipette with DEPC water to remove any clogged particle.
2) Loose tubing: In rare cases one of the tube connections can get loose. Simply attach the tubing to the connector to solve the issue.

For advanced troubleshooting involving the electronic please send an email to the developer: tirtha_banerjee@u.nus.edu; dbstdb@nus.edu.sg

Buffer composition

30% probe hybridization buffer

30% formamide

5× sodium chloride sodium citrate (SSC)

9 mM citric acid (pH 6.0)

0.1% Tween 20

50 µg/mL heparin

1× Denhardt's solution

2.5% dextran sulfate

For 40 mL of solution

12 mL formamide

10 mL of 20× SSC

360 µL 1 M citric acid, pH 6.0

40 µL of Tween 20

200 µL of 10 mg/mL heparin

800 µL of 50× Denhardt's solution

4 mL of 25% dextran sulfate

Fill up to 40 mL with milliQ/DEPC H2O

30% probe wash buffer

30% formamide

5× sodium chloride sodium citrate (SSC)

9 mM citric acid (pH 6.0)

0.1% Tween 20

50 µg/mL heparin

For 40 mL of solution

12 mL formamide

10 mL of 20× SSC

360 µL 1 M citric acid, pH 6.0

40 µL of Tween 20

200 µL of 10 mg/mL heparin

Fill up to 40 mL with milliQ/DEPC H2O

Amplification buffer

5× sodium chloride sodium citrate (SSC)

0.1% Tween 20

2.5% dextran sulfate

For 40 mL of solution

10 mL of 20× SSC

40 µL of Tween 20

4 mL of 25% dextran sulfate

Fill up to 40 mL with milliQ/DEPC H2O

5× SSCT

5× sodium chloride sodium citrate (SSC)

0.1% Tween 20

For 40 mL of solution

10 mL of 20× SSC

40 µL of Tween 20

Fill up to 40 mL with milliQ/DEPC H2O

25% dextran sulfate

For 40 mL of solution

10 g of dextran sulfate powder

Fill up to 40 mL with milliQ/DEPC H2O

Detergent Solution

1.0% SDS

0.5% Tween 20

Tris-HCl (pH 7.5)

1.0 mM EDTA (pH 8.0)

150.0 mM NaCl

For 50 mL of Solution

5.00 mL 10% SDS (filtered)

1.25 mL Tween 20

2.50 mL 1M Tris-HCl, pH 7.5

0.10 mL 0.5 M EDTA, pH 8.0

1.50 mL 5 M NaCl

Fill up to 50 mL with milliQ/DEPC H2O

1x PBST

For 50 ml of Solution

5ml 10x PBS

50 µL Tween 20

5X SSCT

For 40 ml of solution

10 ml 20x SSCT

40 µL of Tween 20.

List of modified buffers

Hybridization buffer

20% EC hybridization buffer:

Composition: 20% ethylene carbonate (Cat No. E26258-3KG), 5× sodium chloride sodium citrate (SSC), 12 mM citric acid (pH 6.0) (Cat No. 251275-100G), 0.5% Tween 20 (Cat No. P1379-100ML), 100 µg/mL heparin (Cat No. H3393-50KU), 1.2× Denhardt's solution (Cat No. D6001-50G), 2.5% dextran sulfate (Cat No. D6001-50G), 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G).

For 50 mL of solution:

Mix in a 50 ml tube 10 mL ethylene carbonate (Cat No. E26258-3KG), 12 mL of 20× SSC, 400 µL 1 M citric acid (pH 6.0 (Cat No. 251275-100G), 200 µL of Tween 20 (Cat No. P1379-100ML), 400 µL of 10 mg/mL heparin (Cat No. H3393-50KU), 1000 µL of 50× Denhardt's solution (Cat No. D6001-50G), 5 mL of 25% dextran sulfate (Cat No. D6001-50G), 100 µl of 500mM EDTA (pH8.0) (Cat No. E9884-100G). Fill up to 50 mL with DEPC (Cat No. D5758-25ML) H_2O.

Wash buffer

20% EC wash buffer:

Composition: 20% ethylene carbonate (Cat No. E26258-3KG), 5× sodium chloride sodium citrate (SSC), 12 mM citric acid (pH 6.0) (Cat No., 0.5% Tween 20 (Cat No. P1379-100ML), and 100 µg/mL heparin (Cat No. H3393-50KU).

For 50 mL of EC wash solution:

Mix in a 50 ml tube 10 mL ethylene carbonate (Cat No. E26258-3KG), 10 mL of 20× SSC, 400 µL 1 M citric acid (Cat No.), pH 6.0, 200 µL of Tween 20 (Cat No. P1379-100ML), 400 µL of 10 mg/mL heparin (Cat No. H3393-50KU). Fill up to 50 mL with DEPC (Cat No.) H_2O.

Note: The use of ethylene carbonate accelerates the overall FISH hybridization time. Previous reports have suggested use of 15-20% ethylene carbonate as an alternative accelerates DNA-FISH experiments (Kalinka et al., 2020; Matthiesen and Hansen, 2012). 10% ethylene carbonate has also been used in hybridization mixture and wash buffer in MERFISH experiments (Fang et al., 2023; Moffitt et al., 2016a). The buffer presented in this study will freeze in 4°C. Kindly thaw before starting the experiment or prepare fresh.

Imaging Buffer:

Composition: 70% glycerol (Cat No. G7893-500ML) with 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G). For 50 ml solution in a 50ml tube add 35ml 100% glycerol and 100 µl of 500 mM EDTA. Top up to 50 ml using DEPC water, and store at room temperature for up to 2 months.

Permeabilization Solution:

Composition: 4.0% Ammonium lauryl sulfate (Cat No. 09887-250ML), 5.0% Tween 20 (Cat No. P1379-100ML), Tris-HCl pH 7.5 (Cat No. 10812846001), 1.0 mM EDTA pH 8.0 (Cat No. E9884-100G), and 200.0 mM NaCl (Cat No. S9888-25G).

For 50 mL of Solution:

Mix in a 50 ml tube 10.00 mL 20% Ammonium lauryl sulfate (Cat No. 09887-250ML) (filtered), 2.50 mL Tween 20 (Cat No. P1379-100ML), 2.50 mL 1M Tris-HCl (Cat No. 10812846001), pH 7.5, 0.10 mL 0.5 M EDTA (Cat No. E9884-100G) pH 8.0, and 2.00 mL 5 M NaCl (Cat No. S9888-25G). Fill up to 50 mL with DEPC H_2O.

Note: The use of ammonium lauryl sulfate provides an alternative to the harsh sodium dodecyl sulfate (SDS) for permeabilization of the probes. Store at RT or 4°C for up to 2 months.

Mounting Media:

Composition: 70% glycerol (Cat No. G7893-500ML) with 1.0mM EDTA (pH 8.0) (Cat No. E9884-100G). For 50 ml solution in a 50 ml tube add 35ml 100% glycerol and 100 µl of 500 mM EDTA. Top up to 50 ml using DEPC water, adjust the pH to 7.8, and store at room temperature for up to 2 months.

Robot cleaning solution (20 ml)

Composition: 2% SDS (Cat No. 436143-25G), 1% Sodium dichloroisocyanurate (Cat No. 218928-25G) and 1% NaOH (Cat No. 221465-25G).

Alternatively, prepare 20ml of solution by mixing 5ml RNaseZap (ThermoFisher, Cat No. AM9780) and 15ml DEPC water.

Probe preparation

Preparing the primary stock solution (10 pairs of probes: 5µM final volume)

Up to 10 pairs of oligonucleotides were ordered from IDT for one gene, each at a concentration of 100 µM. 100 µl of oligos from each tube were mixed to form a master mix with a final probe concentration of 5 µM in a 2ml microcentrifuge tube (Labselect, Cat No. MCT-001-200). Three to four genes with different hairpin amplifiers (Molecular Instruments) can be designed and tested in a single round of HCR (Choi et al., 2018). The number of genes tested in each round depends on the number of laser lines in the confocal being used. The stock mix of primary probes are stored at -20°C.

Application for primary probe design: https://tdblab.github.io/hcrprobedesigner/hcr_22.1.html

Prepare the 20% EC hybridization buffer with probes complementary to the target RNA

Preparation of DNA oligo mix: Add 10 µl of each of the 5 µM DNA oligo mix and adjust the volume to 1000 µl in 20% EC hybridization buffer in 1.5ml microcentrifuge tube (Labselect, Cat No. MCT-001-150).

Prepare the secondary probes with fluorescent tags

Add 5 µl of each H1 and H2 hairpins (Molecular Instruments) separately in 150+150 µl of Amplification buffer in two 200 µl PCR tubes.

Heat at 95°C for 90 secs in a thermocycler and cool down at room temperature in a dark environment for 30 mins. Mix the two tubes together in a 1.5 ml microcentrifuge tube (Labselect, Cat No. MCT-001-150) and use.

Note: The secondaries can be used multiple times. After the reaction, collect them from the secondary collection tube and store at -20°C. For repeated use, heat the secondary probes in amplification buffer at 42°C for 30 mins before use.

Fixation, permeabilization and sample steps

1) Dissect the tissue in 1x PBS at room temperature.

2) Transfer the tissue in 500 µl 1x PBST supplemented with 4% formaldehyde and fix for 30-60 mins at room temperature (RT) with shaking. Smaller fixation time is required for thinner tissues while longer time is required for thicker tissues.

4) Wash the tissue 3 times with 500 µl 1x PBST (3mins each) at RT.

5) Add 500 µl of detergent solution or alternative permeabilization buffer and leave the tissue for 30 mins at 37°C.

6) Wash 3 times with 500 µl 1x PBST (3 mins each) at RT.

7) Wash 2 times with 500 µl 5x SSCT (3mins each) at RT.

8) Transfer the sample to a 3.5mm confocal dish (SPL, Cat No. 240202783).

9) Transfer the tissue to 500 µl 30% probe hybridization buffer or 20% EC hybridization buffer at RT.

Note: Tissues can be stored in 30% probe hybridization buffer at 4°C for 8 weeks without any significant loss of signal. Do not store samples in 20% EC hybridization buffer as the solution will crystallize at 4°C.

10) Add all the buffers in the probe module plate in Multiplexer v1 system and press the respective button.

Reference

Banerjee, T. Das, Raine, J., Mathuru, A. S. and Chen, K. H. (2024). Spatial mRNA profiling using Rapid Amplified Multiplexed-FISH (RAM-FISH). bioRxiv. https://doi.org/10.1101/2024.12.06.62719

Original protocol

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction : multiplexed , quantitative , sensitive , versatile , robust. Development (Cambridge), 1. https://doi.org/10.1242/dev.165753

Bruce, H. S., Jerz, G., Kelly, S., McCarthy, J., Pomerantz, A., Senevirathne, G., Sherrard, A., Sun, D. A., Wolff, C., & Patel, N. H. (2021). Hybridization Chain Reaction (HCR) In Situ Protocol. September, 10–13. https://doi.org/10.17504/protocols.io.bunznvf6